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Original article

Impact of short-term overfeeding of saturated or unsaturated fat or sugars on the gut microbiota in relation to liver fat in obese and overweight adults

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SUMMARY

Backgrounds & aims: Intestinal microbiota may be causally involved in the pathogenesis of non-alcoholic fatty liver disease (NAFLD). We aimed to study the effect of short-term overfeeding on human gut microbiota in relation to baseline and overfeeding-induced liver steatosis. We also asked whether the baseline microbiota composition is associated to the overfeeding-induced increase in liver fat.

Methods: In a randomized trial, 38 overweight and obese subjects were assigned to consume an excess of 1000 kcal/day of diets rich in either saturated fat, unsaturated fat, or simple sugars for 3 weeks. Fasting blood samples and ^{1}H-MR spectroscopy were used for extensive clinical phenotyping as previously reported (PMID: 29844096). Fecal samples were collected for the analysis of the gut microbiota using 16S rRNA amplicon sequencing, imputed metagenomics and qPCR. Microbiota results were correlated with dietary intakes and clinical measurements before and during overfeeding.

Results: The overall community structure of the microbiota remained highly stable and personalized during overfeeding based on between-sample Bray–Curtis dissimilarity, but the relative abundances of individual taxa were altered in a diet-specific manner: overfeeding saturated fat increased Proteobacteria, while unsaturated fat increased butyrate producers. Sugar overfeeding increased *Lactococcus* and *Escherichia coli*. Imputed functions of the gut microbiota were not affected by overfeeding. Several taxa affected by overfeeding significantly correlated with the changes in host metabolic markers. The baseline levels of proteobacterial family *Desulfovibrionaceae*, and especially genus *Bilophila*, were significantly associated to overfeeding-induced liver fat increase independently of the diet arm. In general, limited overlap was observed between the overfeeding-induced microbiota changes and the liver fat-associated microbiota features at baseline.

Conclusions: Our work indicates that the human gut microbiota is resilient to short-term overfeeding on community level, but specific taxa are altered on diet composition-dependent manner. Generalizable microbiota signatures directly associated with liver steatosis could not be identified. Instead, the carriage of *Bilophila* was identified as a potential novel risk factor for diet-induced liver steatosis in humans.

Clinical trial registry number: NCT02133144 listed on NIH website: ClinicalTrials.gov.

1. Introduction

NAFLD is closely associated with obesity and the metabolic syndrome [1] but its pathogenesis remains elusive [2]. Due to the physiological proximity of the gut to the liver, the intestinal microbiota is highly relevant for NAFLD [3]. Metabolic endotoxemia, characterized by increased circulating levels of LPS derived from Gram-negative bacteria, triggers pro-inflammatory pathways

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leading to insulin resistance in mice [4]. Transplantation studies in rodents have suggested a causal role for the gut microbiota in NAFLD development [5,6]. Yet in humans, there is little consensus on the existence of a specific dysbiotic microbiota pattern in NAFLD [7] and evidence of a causal relationship between microbiota and NAFLD is limited by the cross-sectional nature of most human studies [2]. In addition, there is substantial heterogeneity in the methods used for clinical phenotyping and analysis of microbiota. Dietary intervention studies targeting the gut microbiota in humans in relation to NAFLD or other metabolic diseases have focused on either healthy, lean subjects [8] or obese subjects undergoing weight-loss programs [9–11]. No studies have examined effects of a hypercaloric intervention on gut microbiota in NAFLD in obese and overweight individuals.

The Western diet is rich in fat and simple sugars [12]. Rodent studies have shown that not only the amount but also the type of dietary fat affects the gut microbiota [13,14]. In C57BL/6J mice fed for 8 [13] weeks with high-fat diets (45E% fat) containing either saturated palm oil, olive oil or safflower oil, only the palm oil diet altered the fecal microbiota [13]. During 16-week exposure, all test diets were found to have a significant effect on the cecal microbiota [14]. Both studies reported high intake of saturated fat to be the strongest stimulus for fat accumulation in the liver [13,14]. While distinct effects of unsaturated fat or saturated fat on host physiology have been reported [15,16], it is unclear whether the human gut microbiota responds differently to a high intake of unsaturated fats or saturated fats or carbohydrates. It has also been shown that the response of host physiology to dietary intervention depends partly on the baseline microbiota configuration [17–20].

We recently reported a detailed description of phenotypic alterations and the metabolic pathways involved after 3 weeks of overfeeding of saturated fat, unsaturated fat or simple sugars in obese and overweight individuals, and showed that the diet rich in saturated fats led to the largest increase in the liver fat content [16]. In the present study, we studied the impact of each of these overfeeding regimes on gut microbiota composition and, predicted functions as well as the quantities of total bacteria and butyrate producers. In addition, we studied the relationship between the gut microbiota and nutritional and clinical parameters both at baseline and during overfeeding, and specifically asked whether the baseline microbiota composition was associated to the overfeeding-induced increase in liver fat.

2. Materials and methods

The study was ancillary to an intervention registered at ClinicalTrials.gov as NCT02133144. The study protocol was approved by the Medical Ethical Committees of the Hospital District of Helsinki and Uusimaa and Helsinki University Central Hospital. All volunteers provided an informed, written consent.

2.1. Study design, participants and clinical phenotyping

The exclusion criteria, subject characteristics, study design, diets and clinical phenotyping of the participants have been described in detail in the article reporting the clinical outcomes of the study [16]. In brief, 38 overweight and obese subjects (age 48 ± 2 years, body mass index (BMI) 31 ± 1 kg/m^2) were randomized into three groups to consume an excess of 1000 kcal/day of diets rich in either saturated fat (SAT, 59E% fat; N = 14), unsaturated fat (UNSAT, 60E% fat; N = 12), or simple sugars (CARB, 24E% fat; N = 12) for 3 weeks. Detailed diet compositions have been reported earlier [16]. At baseline, 12 out of 38 participants had NAFLD, defined as liver fat >5.56% by ^{1}HMRS as in the Dallas Heart Study [21]. Fasting blood samples were collected before and after the intervention and the subjects were extensively phenotyped for parameters such as liver fat content by ^{1}HMRS, insulin sensitivity, pathways of intrahepatocellular triglyceride synthesis (lipolysis and de novo lipogenesis) and blood biochemistry (liver enzymes, fasting glucose, free fatty acids, insulin, lipids) [16]. The overfeeding period was followed by a weight-loss period to restore the participants' body weight and liver fat to their original states. Fecal samples were collected for microbiota analysis at baseline, after the intervention and at follow-up approximately 2 months after the intervention. Clinical phenotyping and dietary assessment [16] were not performed in the weight-loss period. Therefore, the analyses focus on the samples taken at baseline and after overfeeding; the follow-up microbiota sample was in addition used to assess beta-diversity of the microbiota over time.

2.2. 16S rRNA gene amplicon sequencing and assessment of metabolic endotoxemia

Fecal DNA extraction, library preparation, Illumina MiSeq sequencing of hypervariable V3–V4 regions of the 16S rRNA gene and sequencing data preprocessing were performed as previously described [16]. Lipopolysaccharide binding protein (LBP) and soluble cluster of differentiation 14 (sCD14) in serum were measured using ELISAs (R&D Systems, Minneapolis, MN, USA) [16].

2.3. Quantification of total bacteria and butyrate-producing capacity by qPCR

Total bacteria and butyrate production capacity were performed by qPCR using a BioRad iCycler iQ thermal cycler system (BioRad, Hercules, CA) with HOT FIREPol® EvaGreen® qPCR Mix Plus (Solis BioDyne, Tartu, Estonia). Total bacteria were quantified [22] with universal primers as previously described [23]. The 10-log-fold standard curves ranging from 10^2 to 10^7 copies were produced using the full-length amplicons of 16S rRNA gene of *Bifidobacterium longum*. For quantification of butyrate production capacity of the microbiota, the butyryl-CoA:acetate CoA-transferase gene was quantified by qPCR as described [24], and the output values were converted based on comparative Ct method [25]. All qPCR assays were performed in triplicate. Precautions were taken to ensure that the data from each triplicate fall within 0.5 threshold cycle (Ct), and clear outliers (>2 standard deviations) were removed before calculating average Ct of each sample. There was no detectable amplification arising from non-template controls in any of the assays. The amplification efficiencies of all qPCR assays ranged from 91% to 98%.

2.4. Data analysis and statistics

Sequencing data were analyzed and visualized using R package *mare* [26], which implements tools from e.g. USEARCH [27] and R package *vegan* [28], and has been optimized for the analysis of microbiota count data. To account for the varying sequencing depth, the number of reads per sample was used as an offset in all statistical models. Microbiota richness and Shannon diversity index were estimated using vegan's *diversity* command. Bray–Curtis dissimilarity, quantifying the compositional dissimilarity between different samples, was used as the between-sample distance metric to estimate beta-diversity. Permutational multivariate analysis of variance (*adonis* function in the *vegan* package) with Bray–Curtis dissimilarities was used to identify factors contributing to the variation in microbiota composition. At baseline, variation in the microbiota was significantly associated to habitual intake of insoluble fibre (7%, p = 0.02 in permutational multivariate ANOVA) and age (6%, p = 0.01), while BMI or body weight were not associated

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with the variation in the microbiota or predicted functions ($p > 0.05$). Hence, all comparisons for the baseline microbiota composition were performed with and without adjustment for insoluble fibre intake and age. The significantly differentially abundant taxa identified in both models were consistent; p-values for the unadjusted model are reported. *Mare* functions "*GroupTest*" and "*CovariateTest*" implementing generalized linear models using negative binomial distribution from *MASS* [29] were used to evaluate the differences in the relative abundance of common bacterial genera, families and phyla (>0.01% abundance; > 30% prevalence) between different groups and to assess associations between the abundances of common bacterial taxa and clinical and dietary variables, respectively. Heat-map visualization of baseline associations between bacterial genera and clinical and nutritional variables and their statistical significance was done using the R function *CorrelationMap* in *mare* that implements Spearman correlation test. Functional prediction and annotation were made using Parallel-META 3 [30]. The comparison of differentially abundant pathways based on Kyoto Encyclopedia of Genes and Genomes (KEGG) [31] between NAFLD and non-NAFLD subjects was performed using LEfSe analysis [32] under the condition $\alpha = 0.01$ with an LDA score of at least 2.5. For the exploratory responder analyses, we stratified all subjects based on their liver fat increment during overfeeding. Responders are defined as subjects with any increase in the liver fat (N = 29), and non-responders as subjects whose liver fat content measured by ^{1}HMRS did not increase (N = 9). P-values were adjusted by the Benjamini-Hochberg method for multiple testing in all comparative and correlation tests involving count data for multiple taxa. P-values < 0.05 and FDR-adjusted p-values (adj. p) < 0.2 were considered significant.

Anthropometric and metabolic data are presented as the means with SDs for normally distributed variables and as medians (quartiles 1–3) for non-normally distributed variables. Non-count data (anthropometric and metabolic parameters, microbiota diversity and richness) were analyzed with Wilcoxon signed-rank test (comparison of variables before and after overfeeding) and Kruskal–Wallis test (comparison of variables between three diets). Spearman's test and partial Spearman's test were performed for normal and adjusted correlations of non-count variables, respectively. All significant correlations underwent visual inspection when applicable to eliminate statistically significant correlations driven by few extreme values. Only statistically significant and visually validated results are reported.

3. Results

3.1. Cross-sectional analysis of the relationship between clinical parameters and gut microbiota at baseline

Firmicutes, mainly the families *Ruminococcaceae* and *Lachnospiraceae,* vastly dominated the subjects' microbiota (mean 86%, range 73%–97%), followed by Bacteroidetes (8%, 0%–23%) and Actinobacteria (6%, 0%–20%). Verrucomicrobia and Proteobacteria represented on the average less than 1% of the microbiota.

The microbiota of the subjects with NAFLD (N = 12) differed from the rest (N = 26), as they clustered separately in ordination space using principal coordinates analysis (PCoA) based on the Bray–Curtis distance both compositionally (Fig. 1A) and based on predicted functions (Supplementary Fig. 1A), explaining 7% ($p = 0.007$) and 10% ($p = 0.02$) of their variation in permutational multivariate ANOVA. Differential abundance testing revealed that the microbiota in subjects with NAFLD was enriched in *Blautia* and an unclassified genus of *Lachnospiraceae*, whereas phylum Bacteroidetes and genera *Bacteroides, Alistipes and Clostridium* were reduced in subjects with NAFLD (Fig. 1B). The analysis on the inferred gene families from KEGG Orthology groups showed that genes related to cell membrane transport were overrepresented in the NAFLD gut microbiota, while genes associated with glycan biosynthesis and metabolism were enriched in subjects without NAFLD (Supplementary Fig. 1B).

Genera *Bacteroides, Alistipes* and *Clostridium* correlated significantly inversely with liver fat (Fig. 1C). In addition to liver fat, *Alistipes* was negatively associated with several other traits specific to metabolic derangements, such as plasma triglycerides and plasma glucose. *Blautia* was associated with lower intake of total and insoluble dietary fibre as well as poly- and monounsaturated fat. The Firmicute to Bacteroidetes ratio was significantly higher in the subjects with NAFLD ($p = 0.003$, Supplementary Table 1) and positively associated with liver fat ($R^2 = 0.29$, $p < 0.001$). This relationship remained significant after controlling for age and insoluble fibre intake ($p < 0.001$). No significant associations were identified between the predicted functional modules of the microbiota and host variables including liver fat. Taken together, increased relative abundances of *Blautia* and unclassified *Lachnospiracecae* as well as a higher Firmicute to Bacteroidetes ratio were associated to increased liver fat content at baseline.

3.2. Effects of overfeeding on intestinal microbiota

At baseline, the SAT (N = 14), UNSAT (N = 12) and CARB (N = 12) groups were comparable with respect to liver fat, anthropometric, habitual dietary and blood biochemical characteristics [16]. Also baseline microbiota diversity (SAT = 11.9 (8.3–13.5); UNSAT = 11.4 (9.2–13.4); CARB = 11.1 (8.6–13.2), richness (SAT = 81 (75–86); UNSAT = 78 (70–86); CARB = 76 (64–92), and abundances of bacterial phyla and genera were comparable across the three groups.

The 3-week period of overfeeding significantly and similarly increased BMI in all groups (Supplementary Table 2). The increase in the liver fat was greater in the SAT group compared to UNSAT and CARB groups ($p = 0.03$). Moreover, insulin resistance, activities of liver enzymes, concentrations of plasma HDL and LDL cholesterol as well as the LBP to CD14 ratio as a marker of endotoxemia increased significantly only in the SAT group ($p < 0.01$). The intervention compliance was ensured by assessment of dietary profiles and fatty acid composition of fasting plasma VLDL-TG [16].

Beta-diversity, assessed using PCoA plots based on between-sample Bray–Curtis distances, showed strong clustering of the microbiota samples by the individual (Fig. 2), indicating stability of the individualized microbiota compositions throughout the trial. The variance of the bacterial community compositions was predominantly explained by the individual (72%, $p = 0.001$; Fig. 2), while the microbiota composition (Supplementary Fig. 2A–C) or predicted functions (data not shown) measured with permutational multivariate ANOVA were not associated to the sampling time points or overfeeding diets. No differences were observed on microbiota richness, alpha diversity or Firmicute to Bacteroidetes ratio ($p > 0.05$). Finally, the potential impact of the intervention on the colonic bacterial biomass was estimated based on the qPCR assay for total bacteria, calculated as the number of 16S rRNA genes per gram of stool. The mean bacterial copy number/per gram of stool was not different at baseline and after overfeeding (1.2×10^{12} vs. 1.1×10^{12}) or between the diets after overfeeding (both $p > 0.05$).

3.3. Identification of taxon-specific alterations in response to intervention

We next zoomed into individual taxa to identify specific bacteria that were affected by overfeeding (Table 1). The SAT diet led to a

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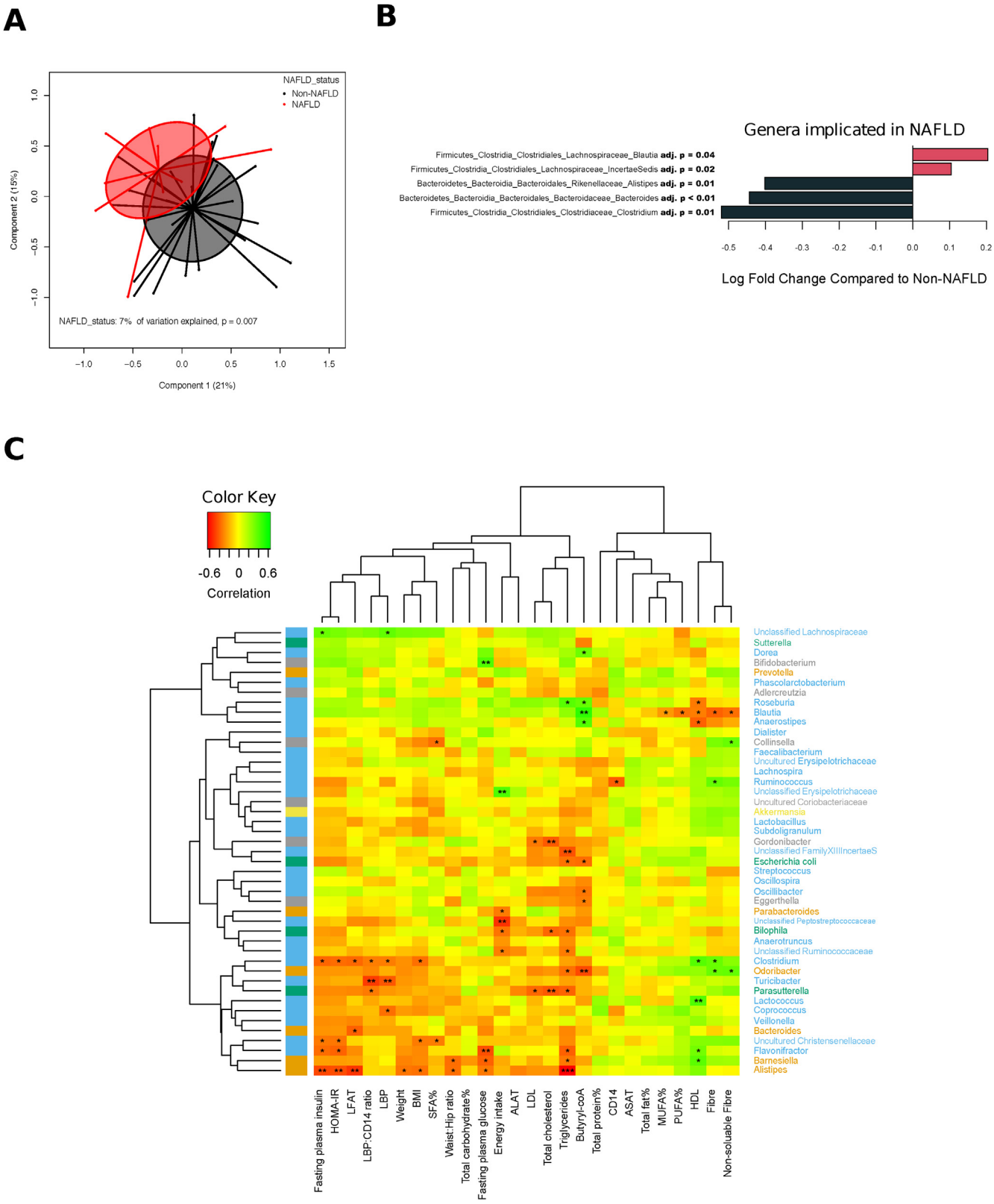

Fig. 1. Baseline microbiota analysis of participants with NAFLD (N = 12) and without NAFLD (N = 26). (A) Principal coordinates analysis (PCoA) plot based on Bray–Curtis distances showing differences in gut microbiota compositions between subjects with NAFLD (red) and without NAFLD (black). (B) Bacterial genera overrepresented in subjects with NAFLD (red) and without NAFLD (black). (C) Heat map displaying baseline associations between gut bacterial genera and clinical or nutritional variables. Statistically significant p-values are noted with an asterisk (*p < 0.05), a double asterisk (**p < 0.01), or a triple asterisk (***p < 0.001). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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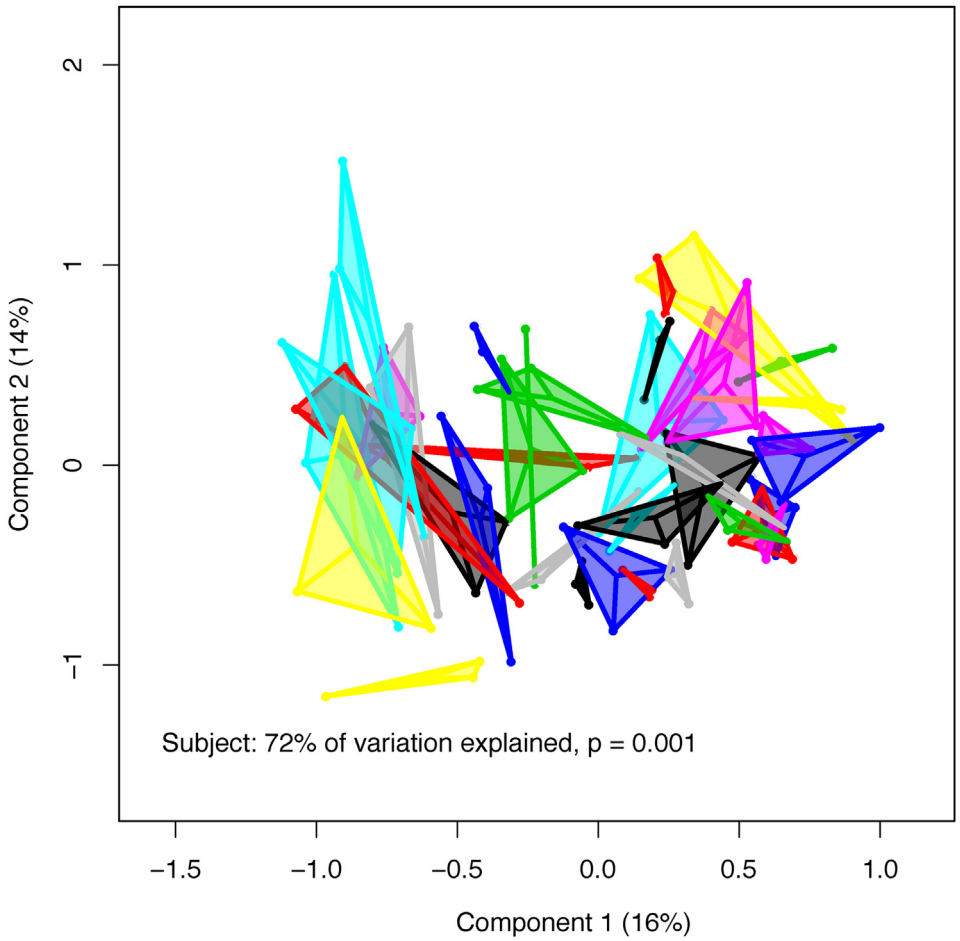

Fig. 2. Principal coordinates analysis (PCoA) plot of the participants' gut microbiota throughout the trial. Samples are colored by the subject and polygons connect the three samples from each participant based on Bray–Curtis distances.

Table 1
Bacterial phyla, families and genera with differential abundances (raw p-values < 0.05 in at least one of the intervention diets) following overfeeding. Fold change is compared to baseline abundance. P-values that remain significant after FDR adjustment are bolded (adj. p < 0.2).

	SAT		UNSAT		CARB		All	
	p-value (adj. p)	Fold change	p-value (adj. p)	Fold change	p-value (adj. p)	Fold change	p-value (adj. p)	Fold change
Phylum								
Proteobacteria	**0.03 (0.14)**	3.7	0.34 (0.52)	1.9	0.56 (0.67)	3.4	**0.01 (0.07)**	2.6
Family								
Desulfovibrionaceae	0.01 (0.28)	2.8	0.06 (0.52)	2.1	0.38 (0.79)	0.5	0.23 (0.79)	1.7
Genus								
Uncultured *Coriobacteriaceae*	0.77 (0.86)	1.2	**0.02 (0.18)**	0.5	0.11 (0.66)	1.1	0.69 (0.9)	0.9
Anaerostipes	0.16 (0.86)	0.8	0.04 (0.22)	0.5	0.41 (0.88)	0.9	**0.03 (0.19)**	0.7
Lachnospira	0.4 (0.86)	1.5	**0.01 (0.12)**	1.7	0.68 (0.94)	0.8	0.15 (0.73)	1.4
Roseburia	0.38 (0.86)	1.2	**0.005 (0.12)**	1.4	0.93 (0.97)	1	0.16 (0.73)	1.2
Unclassified *Ruminococcaceae*	0.68 (0.86)	1.1	**0.03 (0.18)**	1.5	0.47 (0.91)	0.9	0.58 (0.9)	1.1
Lactococcus	0.77 (0.86)	2.2	0.98 (0.98)	0.7	**0.003 (0.14)**	4.1	0.21 (0.79)	1.6
Escherichia coli	0.75 (0.86)	1.5	0.67 (0.91)	2	**0.01 (0.18)**	60.7	0.17 (0.79)	2
Bilophila	0.01 (0.38)	2.8	0.06 (0.67)	2.1	0.38 (0.9)	0.5	0.23 (0.79)	1.7

significant phylum-level increase in the abundance of Proteobacteria ($p = 0.03$, adj. $p = 0.14$). The increase was mainly attributed to *Desulfovibrionaceae* (dominated by *Bilophila* spp.), although not reaching statistical significance after FDR adjustment ($p = 0.01$, adj. $p = 0.28$) because of the limited prevalence of this bacterial family among the study subjects (Supplementary Fig. 3). *Lactococcus* and *Escherichia coli* thrived on the CARB diet (Table 1). The high mean increase (i.e. 60-fold) in the abundance of *E. coli* in the CARB group was mainly attributed to one individual. The UNSAT diet resulted in significant increases in the butyrate producers *Lachnospira, Roseburia* and unclassified *Ruminococcaceae*, and a decrease in uncultured *Coriobacteriaceae* (Table 1).

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3.4. Butyrate-producing capacity of the microbiota

Due to the significantly increased abundances of several butyrate-producing bacteria following the UNSAT overfeeding (Table 1), we estimated the overall butyrate-producing capacity of the microbiota by quantifying the butyryl-CoA:acetate CoA-transferase gene (butyryl-CoA) using qPCR. At baseline, the abundance of the butyryl-CoA gene correlated significantly and positively with the collective relative abundance of dominant butyrate producing genera *Blautia*, *Subdoligranulum, Faecalibacterium, Anaerostipes, Butyrivibrio, Coprococcus* and *Roseburia/Eubacterium rectale* [24] (Supplementary Fig. 4). Butyryl-CoA gene abundance did not change during overfeeding and was uncorrelated with the changes in clinical and nutritional variables.

3.5. Association of baseline microbiota to overfeeding-induced increase in the liver fat

We examined the baseline abundances of common bacterial taxa in all subjects stratified by the clinical outcome, here liver fat increment, as done in previous studies [17,18,33]. There was no difference in the clinical and dietary characteristics between the responders and non-responders at baseline. However, the baseline prevalence and mean abundance of *Desulfovibrionaceae*, especially genus *Bilophila*, were significantly higher in responders i.e. subjects with overfeeding-induced increase in the liver fat (Fig. 3).

3.6. Correlations between the microbiota composition and clinical and nutritional parameters during overfeeding

To parallel the dynamics between the gut bacteria and the host-associated parameters during the intervention, changes in the abundances of common genera were related to changes in key clinical and dietary variables listed in Supplementary Table 2. Subjects from different diets were pooled to increase the statistical power as all diets led to significant increase in the liver fat content (Supplementary Table 2 and [16]). To account for the differing macronutrients between the diet groups during overfeeding, correlations were calculated using models with and without adjustment for changes in percentage of saturated, unsaturated fat and carbohydrate intake. Several genera significantly correlated to changes in host metabolic parameters in unadjusted and/or adjusted model (Table 2). Notably, *Coprococcus* were consistently and significantly associated with lower levels of triglycerides ($p = 0.002$; adj. $p = 0.04$ in the adjusted model). *Lachnospira* (stimulated by UNSAT) and *Lactococcus* (stimulated by CARB) showed negative and positive significant associations with the waist-hip ratio and fasting plasma insulin, respectively, in the adjusted model. An unclassified genus of *Ruminococcaceae* (most closely related to strictly anaerobic butyrate-producing bacterium *Agathobaculum butyriciproducens*), increased by UNSAT, was significantly negatively associated to levels of LDL cholesterol in both models ($p = 0.02$; adj. $p = 0.2$ in the adjusted model). Together, these findings suggest a potential tripartite interaction between diet, gut microbiota and metabolic health.

Blautia that was enriched in individuals with NAFLD at baseline, did not correlate with the change in the liver fat during overfeeding, but showed a significant negative relationship with the intake of mono- and polyunsaturated fat (Table 2). The rest of the taxa that significantly co-varied with host phenotypic changes during overfeeding (Table 2) did not show overlap with bacteria that associated to NAFLD at baseline (Fig. 1).

4. Discussion

By performing an in-depth gut microbiota analysis during overfeeding in obese and overweight individuals, we show that a 3-week energy surplus on top of habitual diet had neglectable community-level effect on the gut microbiota, while a set of diet composition-specific changes in individual bacterial taxa were detected. The overall resilience (i.e. high intraindividual reproducibility of repeated measurements) of the human gut microbiota aligns well with the ample data from previous dietary intervention studies [34]. It is however well known that changes in the abundance of single bacteria can have a major effect on host physiology [35]. In the present study, the abundance of specific bacteria that may exert detrimental or protective diet-dependent effects on the host was altered in a diet-specific manner. Specifically, the enrichment of Proteobacteria in the saturated fat group and butyrate producers in the unsaturated fat group were observed. While the baseline gut microbiota showed specific patterns associated with the NAFLD phenotype, we found little overlap between these bacterial signatures and the bacteria that were significantly altered during overfeeding, as well as between the correlates of microbiota and liver fat or other host metabolic parameters at baseline versus after overfeeding. Instead, our results suggest that the carriage of *Bilophila* is positively associated to increase of liver fat in response to a hypercaloric diet. These findings are discussed in detail below.

At baseline, we found several features in the gut microbiota specific to individuals with NAFLD that have been documented in previous human studies, including depletion of Bacteroidetes [36,37] and increased proportion of *Blautia* [38]. The relative abundance of unclassified *Lachnospiracecae* and the Firmicute to Bacteroidetes ratio were associated with NAFLD in our and other cohorts of NAFLD and metabolic disturbances despite inconsistency in terms of directionality [39,40]. When comparing the predicted functions of the microbiota between subjects with and without NAFLD, the former was enriched with bacterial genes related to cell membrane transport, while glycan biosynthesis and metabolism genes were relatively more abundant in the latter. Previous studies have linked obesity to overrepresentation of bacterial genes related to cell membrane transport, supposedly by increasing the capacity of nutrient uptake by the bacteria [41,42].

The overall community structure of the microbiota remained stable and personalized during overfeeding with SAT or UNSAT. The lack of community-level effects corroborates the few previous studies that have addressed effects of the type and quantity of dietary fat on the human microbiota in healthy subjects or general population. A 7-day overfeeding intervention with whipping cream (45E% of fat, mainly SAT) among 25 lean young German men did not yield consistent changes in the gut microbiota profiles [8]; similarly to a longer-term (18 weeks) moderate alteration in the intake of SAT (13–14 E% vs. 7–8 E%) in a multiethnic cohort with wide age and body mass index range [43]. Finally, no effects on beta diversity were observed after 8 weeks of omega-3 PUFA supplements in 22 healthy British volunteers [44].

Previously we reported the phylum-level increase of Proteobacteria specifically in the SAT group [16]. Here we further analyzed the SAT-induced alternations on finer taxonomic levels, and identified a nominally significant increase in the proteobacterial family *Desulfovibrionaceae* and specifically its genus *Bilophila*. A previous intervention study including 10 healthy subjects found *Bilophila wadsworthia* to increase during an animal-based (SAT-rich) diet and correlate positively with subjects' baseline SAT intake [45]. Three studies in mice [46–48] have also demonstrated a stimulatory effect of saturated fat on the abundance of *Bilophila*. *Bilophila* is extremely bile-tolerant and produces H_2S [49], which is a genotoxin and mucosal barrier-breaker [48,49]. A recent mouse study

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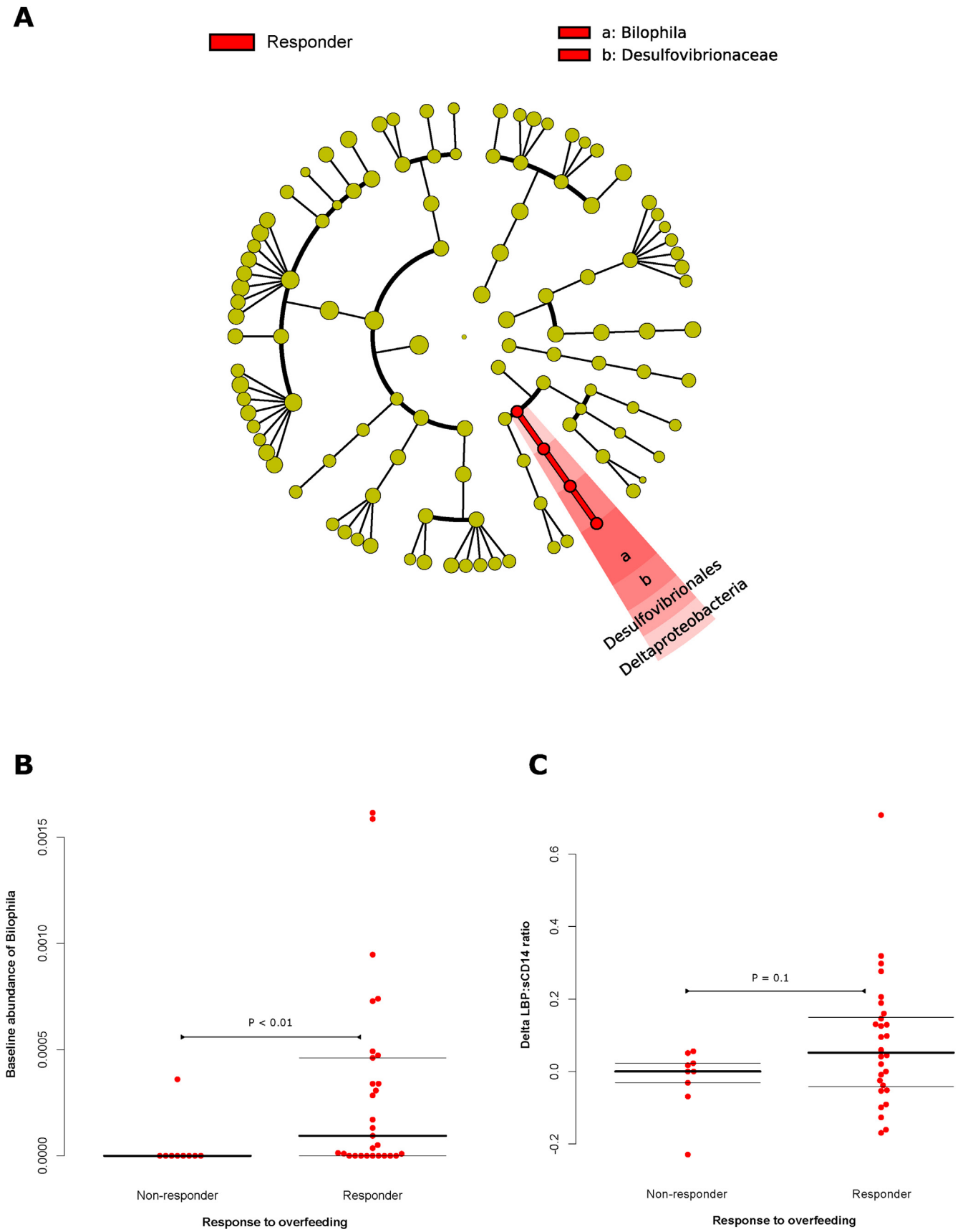

Fig. 3. (A) Cladogram showing differences in taxonomic profiles of the baseline gut microbiota between the liver fat responders and non-responders. (B) Relative abundance of *Bilophila* at baseline. The baseline levels of *Bilophila* were significantly higher in subjects with any increase in the liver fat (N = 29; responders) than in those who did not have increased liver fat during overfeeding (N = 9; non-responders). (C) Intra-individual changes in metabolic endotoxemia (i.e. chronically elevated plasma LPS at levels 10–50 times lower than during septic conditions) during overfeeding. The increase in the LBP to CD14 ratio as marker of metabolic endotoxemia was higher in the responders by trend.

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Table 2
Significant correlations (raw p-values < 0.05 & adj. p values < 0.2) between changes of clinical and nutritional variables and changes of abundances in bacterial genera during overfeeding. Only variables having at least one signifigant association are included in the table and taxa whose abundances were significantly altered by overfeeding (Table 1) are marked with a hash symbol ([a]).

	Estimated effect size[b]	p-value (adj. p)[b]	Estimated effect size[c]	p-value (adj. p)[c]
Waist:Hip ratio				
Lachnospira[a]	NS	NS	−0.04	0.02 (0.2)
Oscillibacter	NS	NS	−0.02	0.01 (0.2)
LDL				
Unclassified *Ruminococcaceae*[a]	−0.02	0.01 (0.18)	−0.02	0.02 (0.2)
Unclassified *Coriobacteriaceae*[a]	NS	NS	0.02	0.02 (0.2)
Dorea	NS	NS	0.03	0.01 (0.2)
Faecalibacterium	NS	NS	−0.15	0.02 (0.2)
Triglycerides				
Coprococcus	−0.02	0.004 (0.1)	−0.02	0.002 (0.04)
Fasting plasma insulin				
Lactococcus[a]	NS	NS	0.01	<0.001 (<0.001)
Fasting plasma blood glucose				
Flavonifractor	0.02	0.007 (0.08)	NS	NS
MUFA intake (%)				
Anaerostipes	−0.01	0.01 (0.09)	–	–
Blautia	−0.03	0.02 (0.09)	–	–
PUFA intake (%)				
Anaerostipes	−0.01	0.01 (0.15)	–	–
Blautia	−0.03	0.02 (0.15)	–	–

NS, not significant; LDL, low-density lipoprotein cholesterol; MUFA, monounsaturated fat; PUFA, polyunsaturated fat.
[a] Significantly altered by overfeeding.
[b] Unadjusted model.
[c] Model adjusted for changes in % intake of saturated, mono- and polyunsaturated fats, and cabohydrates.

shows that *B. wadsworthia* aggravates high fat diet-induced metabolic derangements (including hepatic steatosis) via promoting intestinal barrier dysfunction, inflammation and bile acid dysmetabolism [50]. Interestingly, our exploratory analyses reveal that *Bilophila* was the only taxon that significantly differed at baseline between responders and non-responders for overfeeding-induced liver fat, and hence suggest that higher carriage of *Bilophila* may predispose subjects to overfeeding-induced increase in liver fat.

The UNSAT overfeeding led to the highest number of significant taxon-specific alternations compared to the other two diets. The bacteria up-regulated by UNSAT (*Lachnospira, Roseburia* and unclassified *Ruminococcaceae*) have been associated with leanness and positive metabolic health in our correlative analysis and previous studies [51,52], presumably via butyrate production [52]. These bacteria were previously reported to reversibly increase in healthy subjects supplemented with omega-3 polyunsaturated fat [44]. Therefore, it is unexpected that we found no respective changes in the qPCR-measured butyrate production capacity within the UNSAT group. Nevertheless, the gut microbiota can contribute to host metabolic health and lipid metabolism via multiple pathways [53]. We detected a significant negative correlation between UNSAT-stimulated unclassified *Ruminococcaceae* (related to *A. butyriciproducens*) and serum LDL-cholesterol during overfeeding. *Coriobacteriaceae,* which was significantly reduced by UNSAT and positively associated with LDL, have been linked to poor metabolic health outcomes via a positive correlation with plasma non-HDL cholesterol [54] and enrichment in the obese microbiota [52]. We also identified a strong and significant anticorrelation between changes in the abundance of *Coprococcus* and levels of plasma triglycerides, in line with their negative association in a large cross-sectional study of Finnish men [55].

Similar to high-fat overfeeding, the CARB overfeeding did not alter the global community structure. Instead, we found significant increases in the relative abundances of *Lactococcus* and *E. coli*. Simple sugars, similar to fat that contributed the extra calories in this study, are predominantly digested in the small intestine. Their excessive intake during overfeeding may however make these nutrients accessible to the colonic microbiota. While most colonic bacteria as strict anaerobes cannot utilize fat as an energy source [56], simple sugars escaping the small intestinal absorption are readily fermented by lactic acid bacteria and other facultative anaerobes such as *Lactococcus* and *E. coli* [57], likely explaining their increased abundances during the CARB overfeeding diet. Under anaerobic conditions, *E. coli* and also *Lactococcus* [58] typically switch to mixed-acid fermentation pathway, leading to production of endogenous ethanol that has been implicated in the pathogenesis of NAFLD [59].

Previous human cross-sectional studies have claimed to identify specific NAFLD-associated gut microbiota signatures despite discrepancies in the implicated bacterial taxa [7,60,61]. We attempted to relate the composition and predicted functions of the microbiota to liver fat, other metabolic parameters and dietary variables in both cross-sectional and intervention settings. However, none of the NAFLD-associated microbial signatures we identified at baseline co-varied with the increase of liver fat or changes in related host metabolic parameters as a result of overfeeding. *Blautia*, enriched in subjects with NAFLD at baseline, showed significant anticorrelation with the intake of poly- and monounsaturated fat both at baseline and during overfeeding. The habitual intake of poly- and monounsaturated fat was significantly lower in the subjects with NAFLD ($p < 0.05$; Supplementary Table 1). Thus, the higher abundance in *Blautia* in subjects with NAFLD likely reflects the habitual dietary patterns underlying NAFLD development. In a broader perspective, our findings may imply that the partially inconsistent NAFLD-associated microbial signatures identified in previous cross-sectional studies [7,38,60] are influenced by the dietary patterns specific to the cohorts rather than directly associated with liver fat. These results highlight the importance of taking the habitual diet into account in dietary intervention studies addressing the effects on the gut microbiota.

The main strengths of this study are the type of intervention that is rarely conducted in humans, extensive clinical phenotyping of the volunteers, and the clinical relevance and real-life resemblance of the study design; addressing hepatic steatosis in overweight subjects consuming excess of energy-dense foods. The cohort in the present study was also the largest compared to similar

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interventions [62]. Nevertheless, due to the high individuality of gut microbiota, the sample size per dietary arm is small, limiting statistically significant findings.

In summary, our results confirm the previously identified detrimental association between *Bilophila* and saturated fat intake in metabolic diseases and for the first time demonstrate it in the context of NAFLD. We also identified novel associations between the intake of unsaturated fat intake and gut bacteria that may contribute to compensating the deleterious effects on host physiology generally related to high fat diets. Taken together, these findings reaffirm the importance of diet–microbiota interactions in the process of diet-induced metabolic deterioration.

Authors' contributions

HYJ, PL and SS designed and conceived the clinical study, HY and AS conceived the project, PL and SS acquired the samples and clinical data, CJ processed the fecal samples for microbiota sequencing and performed the qPCR and ELISA experiments, preprocessed and analyzed the sequencing data and performed statistical analyses under the supervision of AS. CJ, AS and HYJ interpreted the results, CJ wrote the manuscript that AS, PL and HY critically revised. All authors approved the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2020.05.008.

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